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Induction of Cytosolic Phospholipase A2 is required for adipose neutrophil Infiltration and hepatic insulin resistance early in the course of high-fat feeding

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Abstract: In established obesity, inflammation and macrophage recruitment likely contribute to the development of insulin resistance. In the current study, we set out to explore whether adipose tissue infiltration by neutrophils that occurs early (3 days) after initiating a high-fat diet (HFD) could contribute to the early occurrence of hepatic insulin resistance and to determine the role of cytosolic phospholipase A2 (cPLA2) in this process. The 3-day HFD caused a significant upregulation of cPLA2 in periepididymal fat and in the liver. A specific antisense oligonucleotide (AS) effectively prevented cPLA2 induction, neutrophil infiltration into adipose tissue (likely involving MIP-2), and protected against 3-day HFD-induced impairment in hepatic insulin signaling and glucose over-production from pyruvate. To sort out the role of adipose neutrophil infiltration independent of cPLA2 induction in the liver, mice were injected intraperitoneally with anti-intracellular adhesion molecule-1 (ICAM-1) antibodies. This effectively prevented neutrophil infiltration without affecting cPLA2 or MIP-2, but like AS, prevented impairment in hepatic insulin signaling, the enhanced pyruvate-to-glucose flux, and the impaired insulin-mediated suppression of hepatic glucose production (assessed by clamp), which were induced by the 3-day HFD. Adipose tissue secretion of tumor necrosis factor- (TNF-) was increased by the 3-day HFD, but not if mice were treated with AS or ICAM-1 antibodies. Moreover, systemic TNF- neutralization prevented 3-day HFD-induced hepatic insulin resistance, suggesting its mediatory role. We propose that an acute, cPLA2-dependent, neutrophil-dominated inflammatory response of adipose tissue contributes to hepatic insulin resistance and glucose overproduction in the early adaptation to high-fat feeding.

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Induction of cytosolic phospholipase A₂ α is required for adipose neutrophil infiltration and hepatic insulin resistance early in the course of high fat feeding

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Running title: The role of adipose cPLA₂ α in hepatic insulin resistance

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In established obesity inflammation and macrophage recruitment likely contribute to the development of insulin resistance. In the present study, we set to explore whether adipose tissue infiltration by neutrophils that occurs early (3 days) after initiating high-fat diet (3dHFD) could contribute to the early occurrence of hepatic insulin resistance, and to determine the role of cytosolic phospholipaseA₂ α (cPLA₂ α) in this process. 3dHFD caused a significant upregulation of cPLA₂ α in peri-epididymal fat and in the liver. A specific antisense oligonucleotide (AS) effectively prevented cPLA₂ α induction, neutrophil infiltration into adipose tissue (likely involving MIP-2), and protected against 3dHFD-induced impairment in hepatic insulin signaling and glucose over-production from pyruvate. To sort out the role of adipose neutrophil infiltration independent of cPLA₂ α induction in the liver, mice were injected intraperitoneally with anti-ICAM-1 antibodies. This effectively prevented neutrophil infiltration without affecting cPLA₂ α or MIP-2, but like AS, prevented impairment in hepatic insulin signaling, the enhanced glucose-to-pyruvate flux, and the impaired insulin-mediated suppression of hepatic glucose production (assessed by clamp), which were induced by 3dHFD. Adipose tissue secretion of TNF α was increased by 3dHFD, but not if mice were treated with AS or ICAM-1 antibodies. Moreover, systemic TNF α neutralization prevented 3dHFD-induced hepatic insulin resistance, suggesting its mediatory role. We propose that an acute, cPLA₂ α -dependent, neutrophil-dominated inflammatory response of adipose tissue contributes to hepatic insulin resistance and glucose overproduction in the early adaptation to high-fat feeding.

INTRODUCTION

Established obesity, particularly if associated with insulin resistance-related morbidities, is characterized by systemic and adipose tissue inflammation (1-3). The complexity of the adipo-cytokines and inflammatory cell types involved in adipose inflammation is constantly increasing, and today most myeloid cell types have been implicated in the process, including macrophages, B-cells, various T-lymphocyte classes and even eosinophils and mast cells (4-6). In contrast to the above, much less is known about adipose tissue and liver adaptation to short-term high fat diet, before overt obesity is present. Metabolically, it appears that hepatic insulin resistance may be a front-line response to short-term (3 days) high-fat diet (3dHFD) (7; 8), representing either "physiological adaptation" and/or an early mal-adaptive response on the causal pathway to obesity-induced whole-body insulin resistance. It currently remains unclear to what degree this early response to HFD involves immune cells in general, and specifically in adipose tissue.

In a previous study we have demonstrated that during the first week of initiating HFD, adipose tissue is infiltrated by neutrophils (9). Adipose tissue protein levels of the neutrophil-specific myeloperoxidase were increased, and correspondingly, by histology an increased number of neutrophils was detected within the parenchyma of adipose tissue (i.e., not restricted to blood vessels). This early appearance of neutrophils in adipose tissue was recently confirmed (10), suggesting that adipose tissue inflammation in obesity largely follows the classical inflammation paradigms of acute versus chronic inflammatory cell infiltrates, predominated first by neutrophils, then lymphocytes in the sub-acute period, and finally by mononuclear macrophages, when inflammation becomes chronic. Yet, the co-occurrence of increased adipose neutrophil infiltration (9; 10) with the early

hepatic insulin resistance (7; 8) begs the question of whether the former phenomenon is causative for the latter.

Cytosolic phospholipase A₂α (cPLA₂α) has received much attention as a key regulator of inflammation. It plays a major role in stimulus-initiated production of eicosanoids (prostaglandins and the chemoattractant leukotrienes) and platelet activating factor (11). In a previous study we demonstrated that cPLA₂α is upregulated in vascular endothelial cells in adipose tissue of mice in response to 3dHFD and that it mediates the elevated expression of the endothelial adhesion molecule ICAM-1 (12) that is utilized for adhesion by neutrophils and monocytes. In addition, cPLA₂α has been demonstrated to regulate superoxide generation by NADPH oxidase activation (13), thus promoting phagocyte-induced oxidative stress. Intriguingly, in humans, even a single exposure to high-fat containing meal induced NADPH activation and inflammatory cascades in circulating leucocytes (14-16). These findings suggest that cPLA₂α could participate in priming/activation of circulating cells upstream in inflammatory cascades that ultimately lead to adipose tissue infiltration by neutrophils, way before obesity has developed. In the present study we set up to reveal the role of cPLA₂α and adipose tissue neutrophil infiltration in the acute adaptation to 3dHFD, with emphasis on whether these early inflammatory responses could underlie the development of hepatic insulin resistance.

RESEARCH DESIGN AND METHODS

Animals and diets. The study was approved by Ben-Gurion University Institutional Animal Care and Use Committee (IL-35-2006), and was conducted according to the Israeli Animal Welfare Act following the guidelines of the Guide for Care and Use of Laboratory Animal (National Research Council, 1996). Male C57BL/6J

mice (Jackson, Maine USA) at 6 weeks of age were fed either a low fat diet (LFD; 6% calories from fat; Harlan Teklad 2018sc) or a high fat diet (60% calories from fat; Research diets #12492), as previously performed (17). At the indicated times mice were euthanized by CO₂ and peri-epididymal fat was dissected out, and immediately fixed in 4% formaldehyde, or snap-frozen and stored in liquid nitrogen until further analyzed. A combination of two oligonucleotides antisenses (tcaaaggtctcattccaca, gctgtcaggggtttag) and their corresponding senses with phosphorothioate modifications on the last 3 bases at both 5' and 3' ends was used as described previously (18). For insulin stimulation, insulin was administered intravenously 5 min prior to killing the mice, and livers or epididymal fat pads were immediately obtained and flash-frozen until analysis. Insulin doses of 0.5 and 2 U/kg gave identical signaling responses. LEAFTM purified anti-mouse Ly-6G antibodies, LEAFTM purified anti-mouse TNF α antibodies (MP6-XT22), LEAFTM purified anti-mouse CD54 antibodies and their isotype control IgG antibodies (BioLegend, San Diego, CA) were used for neutrophils depletion, TNF α neutralization and ICAM-1 neutralization, respectively.

Immunoblot analysis. Epididymal fat were lysed in ice-cold lysis buffer exactly as done earlier (9). Samples were subjected to 7.5% SDS-polyacrylamide gel electrophoresis (for each sample 100 μ g). The resolved proteins were electrophoretically transferred onto a nitrocellulose membrane, blocked in 5% milk in TBS-T (TBS containing 0.1% Tween-20), and incubated overnight at 4°C with either goat anti- mouse myeloperoxidase (MPO) (1:100) (Santa Cruz, CA), anti mouse β -actin (1:10,000), anti-pSer473 Akt (1:1000), anti-Akt (1:1000), anti-pSer9 GSK (1:1000), anti-GSK (1:1000) anti cPLA₂ α (1:200) (Cell signaling, Beverly, MA), anti

5-lipoxygenase (5-LOX) (1:1000) (Cayman, Ann Arbor, USA) anti Cyclooxygenase-2 (COX-2) (1:1000) (Abcam, Cambridge, UK). After being washed with TBS-T (four washes for 15 min each) the membranes were incubated with 1:10,000 dilutions of the respective HRP-conjugated secondary antibodies for 1.5 hr at room temperature and proteins were quantified using video densitometry analysis (ImageGauge version 4.0 Fuji) as described previously (19; 20).

Preparation of neutrophils. Heart blood was withdrawn before sacrifice. Neutrophils, at 95% purity, were obtained by Ficoll-Hypaque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes. Cells were counted and their viability was determined by trypan blue exclusion.

Immunohistochemistry for neutrophil detection - Epididymal fat tissues were embedded in paraffin and sectioned using a Leica microtome (Agentek, Canada). Neutrophil staining by rat anti-mouse monoclonal anti-NIMP-R14 antibody to identify neutrophils (Novus Biological Inc.) was analyzed as done before (9; 13) based on an earlier study (21). Nonspecific reactivity was blocked by incubating the slides with 20% normal rabbit serum for 20 min (avidin-biotin VECTA-STAIN Kit Elite PK 6105; Vector Laboratories, Burlingame CA). Sections were then incubated in 1:10 dilution of anti-NIMP-R14 antibodies in PBS for 1 hr at room temperature, washed, and further incubated with 1:200 dilution of biotinylated anti-rat IgG, followed by avidin-biotin complex/HRP –DAB, which resulted in brown staining of neutrophils. For each treatment a negative control was prepared without the primary antibody. The sections were counter-stained with hematoxylin and analyzed in a blinded fashion using an Olympus BX-60 microscope.

Superoxide generation. The production of the superoxide anion by intact neutrophils was measured as the superoxide dismutase inhibitable reduction of acetyl ferricytochrome c by the microtiter plate technique as previously described (22).

Cytokines and eicosanoids release measurements. Epididymal fat tissue from mice were removed, weight, sliced and incubated for 3 hours in 1 ml medium containing 0.1%BSA. The collected media were measured using commercial ELISA kits for: TNF α (Biolegend, San Diego, CA, USA) 15 μ g tissue for each sample, MIP-2, KC, LTB $_4$, IL-6 (all from R&D systems, Inc. Minneapolis, MN, USA) 150 μ g tissue for each sample. PGE $_2$ levels were determined according to a dextran coated charcoal radioimmunoassay protocol, using PGE $_2$ standard, anti-PGE $_2$ anti-serum (Sigma Israel, Rehovot, Israel) and [3 H]PGE $_2$ (Amersham Biosciences, NJ, USA) exactly as previously described (23).

Pyruvate tolerance test (PTT). PTT was performed by intra-peritoneal injection of 2 g/kg body weight pyruvate in saline after an overnight fast. Blood glucose concentrations were measured via tail bleed before and at times indicated after injection (24) . All glucose measurements were made using a glucometer (Abbott, Alameda, CA).

Homeostasis model index of insulin resistance (HOMA-IR) - was calculated by multiplying fasting plasma insulin concentration (mU/L) with fasting plasma glucose (mmol/l) divided by 22.5 (25-27). Plasma insulin levels were determined by ELISA (ALPCO Diagnostics, Salem, NH, USA)

Glucose clamp studies. Glucose clamp studies in freely moving mice were performed as described (28). Steady-state glucose infusion rate was calculated once glucose infusion reached a near-constant rate to maintain glucose at 5 mmol/l for 20 min. The glucose disposal rate was calculated by dividing the rate of [^3H]glucose infusion by the plasma [^3H]glucose specific activity (29; 30). Endogenous glucose production during the clamp was calculated by subtracting the glucose infusion rate from the glucose disposal rate (29; 30). Insulin-stimulated glucose disposal rate was calculated by subtracting basal endogenous glucose production (equal to basal glucose disposal rate) from glucose disposal rate during the clamp (31).

Statistical analysis. Data are presented as the mean \pm SEM. Statistical significance for comparison between two groups was determined using Student's paired two-tailed t test, and for >2 treatment groups by ANOVA followed by Bonferroni correction or by Newman-Keuls post-hoc test GraphPad Prism version 5.03.

RESULTS

The time-course of development of insulin resistance in response to HFD was determined in liver and in peri-epididymal fat pads by assessing insulin-stimulated phosphorylation of Akt. At different times after initiating HFD, insulin was intravenously injected to the mice 5 min before harvesting livers. Three days after initiating HFD, hepatic insulin-stimulated Ser473-Akt phosphorylation was markedly attenuated (Figure 1A). At this time-point of marked diminution of insulin signaling in the liver, adipose tissue exhibited a normal insulin-stimulated Ser473-Akt

phosphorylation (Figure 1B). Insulin resistance in this tissue required a longer duration of HFD to manifest (not shown). Nevertheless, 3dHFD was already sufficient to induce a statistically significant increase in adipose tissue weight (Figure 1C) and a notable increase in adipocyte cell size (Figure 1D). As reported in a previous study (9) multiple neutrophils were visible between adipocytes, indicating infiltration of the tissue parenchyma. (Figure 1D). Concomitantly, by histology, hepatic lipid content was notably mildly increased in mice on 3dHFD (Figure 1E), as were fasting glucose levels (Table 1). Calculated HOMA-IR values revealed systemic insulin resistance induced by 3dHFD (Table 1).

We hypothesized that adipose tissue neutrophil infiltration induced by 3 days of HFD (9) could participate in the early induction of hepatic insulin resistance. To test this hypothesis, adipose neutrophil recruitment was first prevented using oligo-antisense against cPLA₂ α (AS). This approach was based on previous findings demonstrating: i. the effectiveness of AS administration in inhibiting neutrophil recruitment to the site of inflammation (13); ii. that 3dHFD induces cPLA₂ α up-regulation in adipose tissue (12). One mg/kg AS, 1 mg/kg corresponding (control) sense oligonucleotide, or saline, were intravenously injected to the tail vein of mice one day prior to, and then daily during the 3dHFD. Weight gain was not affected by either AS or sense treatments (Figure 2A), but AS treatment significantly prevented the elevated cPLA₂ α protein expression detected in lysates of adipose tissue of 3dHFD mice (Figure 2B). The AS treatment also significantly prevented the recruitment of neutrophils to the adipose tissue of mice on HFD, as detected by adipose protein levels of MPO (Figure 2C) and by quantitation of neutrophils by NIMP-R14 immunostaining (Figure 2D). To analyze whether AS protected against hepatic insulin resistance, insulin induction of liver phospho-Ser473-Akt or phospho-

Ser9-GSK3 β was assessed (32; 33). AS, but not sense treatment, prevented HFD-induced impairment of insulin-stimulated Akt and GSK-3 β phosphorylation (Figures 2E, 2F), without affecting liver histology (not shown). AS treatment did not affect the normal insulin signaling in the adipose tissue (not shown). At the whole-body metabolic level, 3dHFD was associated with an exaggerated glucose production during a pyruvate tolerance test as compared to LFD mice, and this enhanced pyruvate-to-glucose flux was prevented by AS as compared to the sense-treated mice (Figure 2G). Liver cPLA $_2\alpha$ was also upregulated by 3dHFD, and like in adipose tissue this increased expression was prevented by AS treatment (Figure 3A). However, neutrophils infiltration to adipose tissue seemed to constitute a unique response of this tissue to 3dHFD, as there was no similar elevation in the levels of the neutrophil-selective protein MPO in the liver (Figure 3B). Thus, AS directed against cPLA $_2\alpha$ decreased the elevated expression of this phospholipase in both adipose tissue and the liver, and concomitantly prevented the tissue-selective neutrophil infiltration into adipose tissue, which were induced by 3dHFD. These effects were associated with prevention of impaired hepatic insulin signaling and enhanced pyruvate-to-glucose flux that developed during early adaptation to HFD.

To further support the role of neutrophil recruitment to adipose tissue in inducing liver insulin resistance, and to differentiate its effect from the possible consequences of increased expression of cPLA $_2\alpha$ in the liver, neutrophil recruitment was prevented using anti ICAM-1 antibodies. Mice were injected intra-peritoneally with 100 μ g anti-mouse ICAM-1 antibodies or isotype control IgG antibodies every other day beginning 2 days prior to initiating HFD. Treatment with anti ICAM-1 antibodies, but not with control IgG antibodies, reduced MPO expression in adipose tissue (Figure 4A) and the number of neutrophils as analyzed histologically by NIMP-

R14 (from 11.56 ± 1.2 to 3.49 ± 0.9 neutrophils per high power field, $p < 0.001$), indicating the prevention of adipose neutrophil recruitment, without affecting body weight (Figure 4B). Importantly, this approach to prevent HFD-induced adipose neutrophil infiltration did not attenuate the diet-induced elevation in cPLA $_2\alpha$ protein expression in the liver (Figure 4C). Inhibition of neutrophil infiltration to adipose tissue significantly improved hepatic insulin signaling, as detected by insulin-stimulated p-Akt and p-GSK (Figure 4D, E) without affecting liver histology (not shown) or adipose insulin signaling (not shown), and consistently, alleviated the augmented gluconeogenic flux from pyruvate induced by 3dHFD (Figure 4F). To further confirm that prevention of adipose infiltration by neutrophils improved hepatic insulin sensitivity, hyperinsulinemic-euglycemic clamp studies were conducted. Glucose infusion rate was significantly decreased in HFD-fed mice (Fig. 4G and Suppl. Fig. 1). However, no difference was observed between IgG- and ICAM-treated mice (Fig. 4G and Suppl. Fig. 1). Insulin-mediated inhibition of endogenous (mainly reflecting hepatic) glucose production was significantly reduced in IgG-treated HFD-fed mice (Fig. 4H). In contrast, ICAM-treated HFD-fed mice showed a conserved response to insulin suggesting improved hepatic insulin sensitivity in ICAM-treated compared to IgG-treated HFD-fed mice (Fig. 4H). Of note, three days of HFD did not significantly impact insulin-stimulated glucose disposal rate (mainly reflecting skeletal muscle insulin sensitivity) although a slight decrease in the anti-ICAM-1-treated group was apparent (Figure 4I). High variability in insulin-stimulated glucose disposal rate may have prevented a significant difference in GIR between the two HFD-fed groups (Fig. 4G). These hyperinsulinemic-euglycemic clamp results are consistent with a state of selective hepatic insulin resistance induced by 3dHFD,

which can be alleviated by anti-ICAM-1 antibodies that prevent adipose tissue neutrophil infiltration.

To gain further support for the role of adipose neutrophil infiltration in the induction of hepatic insulin resistance, neutrophil infiltration was prevented by an alternative approach, i.e., by neutrophil depletion using Ly6G-specific monoclonal antibody, as described (34; 35). Mice were injected intra-peritoneally with 150 μ g anti-mouse Ly-6G antibodies or isotype control IgG antibodies one day prior to initiating 3dHFD. Neutrophil depletion was validated by FACS analysis using rat anti-mouse neutrophils antibodies (MCA771F, Serotec), as performed in a previous study (13). Neutrophil depletion significantly improved hepatic insulin responsiveness, as detected by insulin-stimulated p-Akt (Figure 4J) and p-GSK (Figure 4K). Collectively, inhibiting adipose neutrophil infiltration, independent of attenuating cPLA₂ α induction early after initiating HFD, prevented the early diet-induced hepatic insulin resistance and glucose over-production.

We next assessed possible mechanisms related to: i. neutrophils' adaptation to HFD and to their recruitment to adipose tissue; ii. the induction of hepatic insulin resistance in this specific model of short-term hyper-nutrition. Neutrophils' tissue infiltration is a process associated with cellular activation and with specific chemoattractants that direct these cells to the site of inflammation. Indeed, peripheral blood neutrophils from mice on 3dHFD released significantly higher levels of superoxides (Figure 5A), suggesting that neutrophil activation is induced by the diet. Consistent with our previous report (13), the AS treatment attenuated the release of superoxides. Several putative adipose tissue-derived neutrophil chemoattractants are known as potent activators. These factors include the cPLA₂-dependent metabolite LTB₄, previously shown by us to recruit neutrophils to the peritoneal cavity of a

mouse model of sterile peritonitis (13), and the neutrophil-selective chemoattractant IL-8. The IL-8 ortholog in mice is keratinocyte chemoattractant (KC or CXCL1), and MIP-2 (CXCL2), both ligands for the chemokine receptor, CXCR2 (36). Epididymal fat tissue from 3dHFD-fed mice and control (3dLFD) mice expressed similar levels of 5-LOX protein and released similar levels of LTB₄, with no effect on these parameters by AS treatment (Figure 5B,5C). Similarly, secretion of the neutrophil chemoattractant KC was not affected by 3dHFD or by the AS treatment (Fig. 5D). Yet, a significantly increased secretion of MIP-2 from adipose tissue of mice on 3dHFD was detected (Figure 5E, 5F). AS-treated 3dHFD mice secreted significantly lower levels of MIP-2, similar to those of the control mice (Fig 5E), suggesting that cPLA₂α is involved in regulation of MIP-2 production. In contrast, treatment of 3dHFD mice with anti ICAM-1 antibodies did not attenuate MIP-2 secretion (Figure 5F), suggesting that cPLA₂α upregulation, but not the consequential neutrophil infiltration, are involved in adipose tissue MIP-2 production. Jointly these findings suggest a putative role for adipose MIP-2 as a chemoattractant involved in neutrophil recruitment to the epididymal fat tissue early after initiating HFD.

In order to shed light on the mechanisms by which neutrophil infiltration to adipose tissue might induce hepatic insulin resistance, we determined if secretion/expression of PGE₂, COX2, and the pro-inflammatory cytokines IL-6 and TNFα are increased already 3 days after initiating HFD. Both cPLA₂α and COX2 are robustly increased in various tissues during active inflammatory processes, and PGE₂, the metabolite of arachidonic acid, has been recently implicated in insulin resistance (37). Yet, no increase in either COX2 expression or PGE₂ secretion from adipose tissue was observed in 3dHFD compared with control mice (Figures 6A,6B, respectively). There was also no increase in the secretion of IL-6 (Figure 6C), but

TNF α (Figure 6D,6E) was significantly elevated in adipose tissue of mice on HFD, an effect of the diet, prevented by either AS or anti-ICAM-1 antibodies. To further establish the mediatory role of TNF α in hepatic insulin resistance following 3dHFD, TNF α was neutralized, using anti-mouse IgG1 monoclonal antibody, as in (38). Mice were injected intra-peritoneally with 150 μ g anti-mouse TNF α antibodies or isotype control IgG1 antibodies, 1 day prior to initiating HFD and at day 2 of HFD. The elevated serum TNF α levels in HFD mice (8.7 ± 0.9 pg/ml compared with 5.5 ± 0.7 in LFD mice) was significantly ($p < 0.001$) reduced by anti TNF α antibodies treatment (4.5 ± 0.6 pg/ml), but not by control IgG1 antibodies (8.1 ± 0.7 pg/ml). Serum TNF α neutralization significantly improved hepatic insulin responsiveness, as detected by insulin-stimulated p-Akt and p-GSK (Figure 6F, 6G.)

DISCUSSION

The present study shows that hepatic insulin resistance induced by short-term HFD reflects dys-regulated adipose-to-liver axis that is initiated by a cPLA $_2\alpha$ -mediated, neutrophil-predominated, adipose tissue inflammation. Our studies reveal that: **i.** Hepatic insulin resistance develops as early as 3 days after initiating HFD, consistent with recently published studies (7; 8); **ii.** Neutrophils recruitment to adipose tissue is critical for the induction of hepatic insulin resistance in response to 3dHFD. Increased adipose tissue TNF α secretion may constitute a mediator in this response, since both increased TNF α secretion and hepatic insulin resistance were prevented when neutrophil infiltration was attenuated, and neutralizing TNF α significantly prevented hepatic insulin resistance. **iii.** The recruitment of neutrophils to adipose tissue likely involves cPLA $_2\alpha$ -dependent MIP-2 secretion.

Consistent with both our previous (9) and present studies, recruitment of neutrophils to adipose tissue following 3 days of HFD was recently confirmed by others (10), and the role of adipose tissue neutrophils in insulin resistance was also demonstrated. Adipose tissue recruitment of neutrophils is also consistent with a study demonstrating increased leukocyte-endothelial cells-platelet interaction in the microcirculation of visceral adipose tissue in obesity (39). Moreover, in humans, pregnant obese women with preeclampsia exhibited extensive vascular inflammation characterized predominantly by neutrophils, demonstrable in adipose tissue blood vessels (40). The present study, however, offers several novel observations and mechanistic insights: 3dHFD constitutes a unique state predominantly affecting hepatic insulin responsiveness, independent of skeletal muscle and/or adipose tissue insulin resistance that apparently take longer to develop. Under such conditions of "isolated hepatic insulin resistance", we were able to demonstrate that neutrophil recruitment, specifically to adipose tissue (and not to the liver), are necessary for hepatic insulin resistance to develop. Moreover, induction of adipose cPLA₂ α up-regulation is deemed necessary for adipose tissue neutrophil infiltration, and is upstream of an inflammatory pathway involving the up-regulation of the neutrophil chemoattractant MIP-2, the up-regulation of the endothelial adhesion molecule ICAM-1 (12), and the adipose tissue neutrophils-dependent increased production of TNF α . Importantly, although cPLA₂ α is up-regulated also in the liver (as was neutrophil elastase in a recent study (10)), our results assign a role specifically for adipose, and not hepatic, cPLA₂ α up-regulation in the induction of hepatic insulin resistance and glucose over-production: By antagonizing ICAM-1, 3dHFD-induced increase in cPLA₂ α was still present, but adipose tissue neutrophil infiltration prevented, and hepatic insulin resistance was still averted. Thus, we provide

compelling evidence that adipose cPLA₂α-mediated neutrophil infiltration, specifically to adipose tissue, contributes to hepatic insulin resistance in response to acute short-term high fat feeding. It may be speculated that this early inflammatory response to a high-fat containing diet, consistent with studies in human circulating leucocytes (14-16), represents, at least in the short term, a physiological adaptation: isolated hepatic insulin resistance with preserved adipose tissue insulin sensitivity would assist in directing more storage of excess calories in adipose tissue, and less in ectopic sites.

Recruitment of different immune cell types to adipose tissue and consequential changes in cytokines secretion have been documented during the last years, mainly at much later time points of diet-induced obesity. These include CD11c⁺, M1-like macrophages (2; 4; 41; 42) and B and CD8⁺ T-lymphocytes (43-45). However, the recruitment of lymphocytes to adipose tissue was not directly linked with induction of insulin resistance (5). Although Kupffer cells were proposed to mediate the induction of hepatic insulin resistance by 3dHFD (7), another study reported opposite results (8) suggested that while macrophage-mediated tissue inflammation is a key component of chronic obesity-associated insulin resistance, it is not critical for the decrease in insulin sensitivity - at the early adaptation to HFD feeding. We therefore propose that most likely, in the acute response to HFD, neutrophils may well constitute the major driver of adipose tissue inflammation and hepatic insulin resistance.

cPLA₂α was recently suggested to be involved in the storage of lipids in the adipose tissue and liver during prolonged obesity (46) and in 3T3-L1 cells (47). In the present study prevention of cPLA₂α up-regulation by AS treatment in mice on 3dHFD did not interfere with weight gain. Thus, cPLA₂α regulates the adaptation to 3dHFD independent of weight gain or adipocyte hypertrophy. The results of the

present study suggest that MIP-2, a mouse ortholog of human IL-8, signals neutrophils recruitment downstream of cPLA₂ α (Fig. 6). This observation may be also highly relevant for humans in which adipose tissue IL-8 levels were elevated (48-50). MIP-2 has been shown to be one of the major inducible chemokines with the ability to attract neutrophils to the site of inflammation (51). KC and MIP-2 have temporally distinct patterns of expression and cell type-specificity (52). MIP-2 was shown to be produced by resident macrophages and by neutrophils (36; 51). Therefore in response to 3dHFD MIP-2 is likely produced by resident macrophages and not by neutrophils, since MIP-2 production was reduced only with the AS treatment and not with anti-ICAM-1 antibodies treatment, while in both treatments the recruitment of neutrophils to the adipose fat was prevented. As MIP-2 synthesis is TLR-dependent (36) and saturated FFAs act as a naturally occurring ligands of macrophage TLR4 (53), it is possible that elevation of FFA in the intra-abdominal adipose tissue by 3dHFD triggers the production of cPLA₂ α -dependent MIP-2 in resident macrophages, leading to neutrophils recruitment. Thus, cPLA₂ α facilitates neutrophil recruitment by two important steps of the process; the secretion of chemoattractant MIP-2 and the production of the adhesion molecule ICAM-1(12).

We show here that circulating neutrophils are activated in mice on 3dHFD, as shown by the release of superoxides (Fig. 5A). This is consistent with the immediate activation of circulating neutrophils, the release of superoxides, increased expression of NADPH oxidase, and the increased proinflammatory cytokines demonstrated in healthy human subjects exposed to high fat and carbohydrate meal or during a glucose challenge (14-16; 54-57). The activated adipose neutrophil-produced factors that mediate hepatic insulin resistance have yet to be identified. Although neutrophil elastase is a likely candidate (10), it should be remembered that this was demonstrated

at 12 weeks of HFD, when its expression was also elevated in the liver. LTB₄ or PGE₂ were shown to impair insulin signaling (37; 58) and it was recently reported that adipose tissue LTB₄ secretion contributes to elevation of adipose macrophages and T cells at 15 weeks of HFD (59). Yet, they are unlikely to be involved in the response to 3dHFD. Elevated local and circulating levels of TNF α and IL-6 are known to be associated with obesity in both humans and rodent models (60-62) and both cytokines have been shown to induce hepatic insulin resistance (63; 64). While there was no increase in adipose secretion of IL-6 at 3dHFD, here we show that adipose tissue released significantly higher levels of TNF α , and that prevention of neutrophils infiltration to adipose tissue significantly attenuated this response. TNF α blood levels were significantly elevated by short term HFD, consistent with a recent study (8), and neutralizing these elevated TNF α levels prevented the development of hepatic insulin resistance by 3dHFD, further supporting its causal role in the process. In accordance with our results, a much higher increase in TNF α than in IL-6 gene expression in adipose tissue at day 3 of HFD was reported (8). Thus, we propose TNF α as a potential mediator of primed adipose tissue neutrophils in causing hepatic insulin resistance induced by 3dHFD.

In conclusion, we show here that adipose cPLA₂ α has a profound role in initiating hepatic insulin resistance during early response to hyper-nutrition. Adipose cPLA₂ α has a major role in promoting infiltration of activated neutrophils to adipose tissue in short-term HFD probably by increasing the production of the neutrophil chemoattractant MIP-2 and up-regulating ICAM-1. The presence of neutrophils in the adipose tissue induces elevated secretion of TNF α , which likely contributes to the early development of isolated hepatic insulin resistance.

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N.H. researched the data and reviewed the manuscript, O.B., V.E.C. and Y.S. researched the data, S.W., F.I and D.K. conducted and interpreted the clamp studies, A.R. contributed to study conceptualization, results interpretation, and participated in writing and reviewing the manuscript, and R.L. is the guarantor of this work and, as such, design the study and wrote the manuscript, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Legend to Figures

Figure 1: Short term HFD induces liver, but not adipose tissue insulin resistance.

A. Liver insulin resistance was determined by impaired insulin-stimulated phospho-Akt on serine 473 (p-Akt) after i.v. injection of insulin. Representative immunoblot of p-Akt in liver lysates from mice at day 0 up to day 28 on HFD compared with liver lysates of a mouse that was not treated with insulin (-). After densitometry analysis, the ratio of the intensity of the p-Akt band to total Akt (t-Akt) was calculated and presented, as arbitrary units, by the mean \pm SEM of 10 mice studied in 3 independent experiments. **B.** Normal insulin signaling in epididymal fat of 3dHFD mice. A representative immunoblot of p-Akt in epididymal fat tissue lysates from mice on HFD, control mice on LFD at day 3 and mice on day 0. Densitometry analysis were performed as in A. Results are mean \pm SEM of 15 mice studied in 5 independent experiments. **C.** Epididymal fat mass of 10 mice on HFD and 10 mice on LFD at day 3 of the diet show a significant difference - * $p < 0.001$ between the groups. Results are mean \pm SEM of 2 different experiments. **D.E.** Histological sections of epididymal adipose tissues and immunolocalization of neutrophils (shown by arrows) using anti-NIMP-R14 antibodies and hematoxylin counter-staining (amplification magnitude X 400). **(D.)** Histological sections of liver stained by H&E (amplification magnitude X 200) **(E.)** Shown are representative images of 10 mice in each group.

Figure 2. Inhibition of neutrophils recruitment to epididymal adipose tissue by cPLA₂ α -specific antisense oligonucleotide prevents hepatic insulin resistance

Mice were i.v. injected 1 day prior to the initiation of HFD and everyday during the diet with (2mg/kg) antisenses against cPLA₂ α (AS). The HFD control mice were administrated with either 100 μ l saline or 2mg/kg sense (SE) oligonucleotide, and

since there was no difference between the two groups they were combined (SE). Control LFD mice were fed with normal diet for 3 days. Half of the control LFD mice were administrated with 100 μ l saline that did not cause any effect compared with the non-injected mice. The weight and the densitometry of the immunoblot of the individual mice in each group performed in three independent experiments are presented.

A. * $p < 0.001$ between the two groups of mice on HFD and the group of mice on LFD at day 3 of the diet. **B.** A representative immunoblot of cPLA₂ α and the corresponding β -actin protein expression in epididymal adipose tissue lysates of the different groups of mice. The intensity of each cPLA₂ α band was divided by the intensity of the respective β -actin band after quantitation by densitometry, and expressed as arbitrary units. * $p < 0.001$ of control HFD mice (SE) in comparison to HFD mice with AS treatment or LFD mice. **C.** A representative immunoblot and densitometry analyses of MPO (a specific neutrophil marker) and β -actin in epididymal fat from LFD mice, AS treated HFD mice and control mice (SE). The intensity of each MPO band was divided by the intensity of the respective β -actin band after quantitation by densitometry, and expressed as arbitrary units. * $p < 0.001$ in control HFD mice (SE) in comparison to AS treated HFD mice or LFD mice. **D.** The presence of stained neutrophils using mouse anti-neutrophils antibody (NIMP-R14) in sections of epididymal adipose tissues. Results are the mean \pm SEM number per field, derived from 20 individual fields for each mice from 2 independent experiments. * $p < 0.001$ in control HFD mice (SE) in comparison to AS treated HFD mice or LFD mice. **E.F.** For each group of mice, liver lysate of a mouse that was not treated with insulin (-) and lysates from two different mice treated with insulin (+) are presented. Densitometry analysis for p-Akt and p-GSK were performed as in Fig. 1A.

* $p < 0.001$ - significant decrease of control HFD mice (SE) in comparison to HFD mice with AS treatment or LFD mice. **G.** Pyruvate tolerance test after overnight fast 3dHFD mice. The results are the mean \pm SEM of 6 mice in each group performed in two independent experiments. The inset describes the area under the curve (AUC). * $p < 0.05$, ** $p < 0.01$, compared to SE-treated HFD mice (SE) by two-way ANOVA and Bonferroni's post test analysis.

Figure 3: The effect of cPLA₂ α AS treatment in livers of HFD mice

A. A representative immunoblot analysis of cPLA₂ α and the corresponding β -actin protein expression in liver lysates of the different groups of mice. Densitometry analysis for cPLA₂ α was performed as in Fig. 2B. * $p < 0.001$ - significant increase of control HFD mice (SE) in comparison to HFD mice with AS treatment or LFD mice.

B. A representative immunoblot of MPO in liver of LFD mice, control HFD mice (SE) and AS treated HFD mice. Densitometry analysis for MPO was performed as in Fig. 2C. There are no differences between the various groups of mice.

Figure 4: Prevention of neutrophil recruitment to epididymal adipose tissue by anti-ICAM-1 antibodies prevented hepatic insulin resistance in response to 3dHFD

Mice were i.p.. injected (100 μ g/kg) anti-ICAM-1 antibodies 2 days prior to the initiation of HFD and every other day thereafter. The HFD control mice were administrated with either 100 μ l saline or rat IgG2b, isotype control. The weight and the densitometry of the immunoblot of the individual mice in each group performed in three different experiments are presented.

A. A representative immunoblot of MPO in epididymal fat. Densitometry analysis for MPO was performed as described in Fig. 2C. * $p < 0.001$.- Significant increase of control HFD mice and anti IgG treated HFD mice in comparison to HFD mice treated with anti-ICAM-1 antibodies and LFD mice. **B.** Effect of treatment on body weight. * $p < 0.001$ between the groups of mice on HFD and the group of mice on LFD at day 3 of the diet. **C.** A representative immunoblot analysis of cPLA₂ α and the corresponding β -actin protein expression in adipose tissue lysates of the different groups of mice. Densitometry analysis for cPLA₂ α was performed as in Fig. 2B. There is a significant difference * $p < 0.001$ between the groups of mice on HFD compared with LFD at day 3 of the diet. **D.E.** Representative immunoblots and densitometry analyses of phospho-Akt (serine 473) and phospho-GSK (serine 9). For each group of mice, liver lysate of a mouse that was not treated with insulin (-) and lysates from two different mice treated with insulin (+) are presented. Densitometry analysis for p-Akt and p-GSK were performed as in Fig. 1A. * $p < 0.001$.- Significant decrease of control HFD mice and anti IgG treated HFD mice in comparison to HFD mice treated with anti-ICAM-1 antibodies and LFD mice. **F.** Pyruvate tolerance test after an overnight fast. The inset describes the area under the curve (AUC). The results are the mean \pm SEM of 6 mice in each group performed in two independent experiments ** $p < 0.01$, * $p < 0.05$ compared to ICAM-1 antibody-treated HFD mice by two-way ANOVA and Bonferroni's post test analysis. **G.H.I.** Hyperinsulinemic-euglycemic clamp studies with **(G)** glucose infusion rate (GIR), and **(H)** insulin-inhibited endogenous glucose production (EGP) and **(I)** insulin-stimulated glucose disposal rate were conducted in chow-fed (open), IgG-treated HFD-fed (grey) and ICAM-treated HFD-fed (black) mice. (Further clamp results are in supplemental data 1). Results are the means of 4 (chow-fed) or 4 (HFD-fed)

animals. All error bars represent SEM. * $p < 0.05$, ** $p < 0.01$ ANOVA followed by Newman-Keuls post-hoc test. **J.K.** Neutrophil depletion prevented insulin resistance in 3dHFD liver- Representative immunoblots and densitometry analyses of phospho-Akt (serine 473) and phospho-GSK (serine 9). For each group of mice, liver lysate of a mouse that was not treated with insulin (-) and lysates from two different mice treated with insulin (+) are presented. Densitometry analysis for p-Akt and p-GSK were performed as in Fig. 1A. * $p < 0.001$ - Significant decrease of control HFD mice in comparison to neutrophil depleted HFD mice and LFD mice.

Figure 5: Three days of high fat feeding peripheral blood neutrophil and adipose tissue release of MIP-2

A. Spontaneous superoxide production by isolated peripheral blood neutrophils from individual HFD mice and LFD mice at day 3 of the diet performed in three different experiments is presented. * $p < 0.001$ - significant increase of control HFD mice (SE) in comparison to HFD mice with AS treatment or LFD mice. There are no differences between HFD mice with AS treatment and LFD mice.

B. A representative immunoblot of 5-LOX in epididymal fat lysates of LFD mice, control HFD mice (SE) and AS treated HFD mice. Densitometry analysis were performed as for cPLA₂ α in Fig. 2A. Results are mean \pm SEM of 12 mice. **C.D.**

LTB₄ and KC release from adipose tissue explants of the different groups. There are no significant differences between the groups. **E.** MIP-2 release from adipose tissue explants was significantly increased (* $p < 0.001$) in control HFD mice (SE) in

comparison to HFD mice with AS treatment or LFD mice. **F.** MIP-2 release from adipose tissue explants was significantly higher (* $p < 0.001$) in the groups of mice on

HFD compared with the group of mice on LFD at day 3 of the diet.

Figure 6 – TNF α is the possible adipose mediator for the induction of hepatic insulin resistance in response to short-term high fat diet.

A. A representative immunoblot of COX-2 in adipose lysates of LFD mice, control HFD mice (SE) and AS treated HFD mice. Densitometry analysis were performed as for cPLA $_2\alpha$ in Fig. 2A. **B.C.** PGE $_2$ and IL-6 release from adipose tissue explants. No statistically-significant differences between the groups were noted. **D.** TNF α release from adipose tissue explants was significantly increased (* $p < 0.001$) in control HFD mice (SE) in comparison to HFD mice with AS treatment or LFD mice. **E.** TNF α release from adipose tissue explants was significantly increased (* $p < 0.001$) in control HFD mice (SE) in comparison to HFD mice with AS treatment or LFD mice. **F.G.** TNF α neutralization prevented insulin resistance in 3dHFD liver - Representative immunoblots and densitometry analyses of phospho-Akt (serine 473) and phospho-GSK (serine 9). For each group of mice, liver lysate of a mouse that was not treated with insulin (-) and lysates from two different mice treated with insulin (+) are presented. Densitometry analysis for p-Akt and p-GSK were performed as in Fig. 1A. * $p < 0.001$ - Significant decrease of control and IgG treated HFD mice in comparison to TNF α depleted HFD mice or LFD mice.

Table 1**Metabolic changes induced by 3-day HFD**

	LFD	HFD
3d body weight gain, g	0.28 \pm 0.09	2.28 \pm 0.11*
Epididymal adipose tissue weight, g	0.17 \pm 0.01	0.26 \pm 0.01*
Non-fasting serum insulin μ g/l	1.19 \pm 0.2	1.34 \pm 0.3
Fasting blood glucose mg/dl	110 \pm 9.1	136 \pm 11.1**
Fasting serum insulin μ g/l	0.31 \pm 0.03	0.36 \pm 0.04
HOMA-IR	2.1 \pm 0.19	3.0 \pm 0.25**

The results are mea \pm SEM of 10 mice in each group. Mice were fed a normal diet (LFD) or a high-fat diet for 3 days (HFD). * P <0.001 and ** P < 0.05 in HFD compared with LFD.

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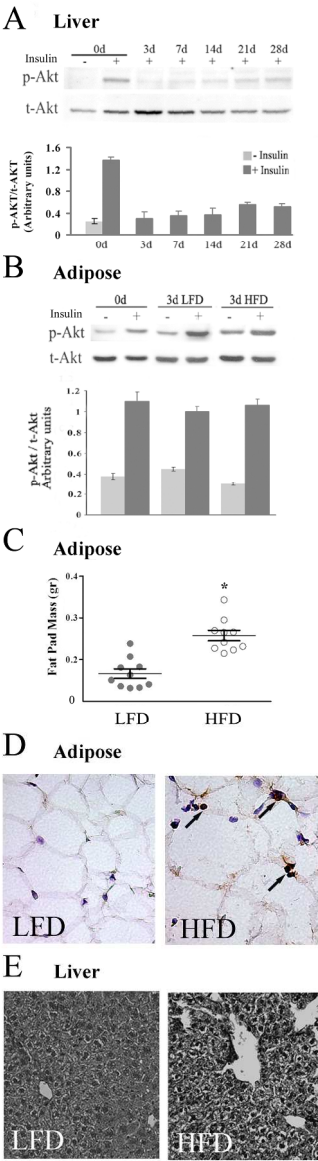
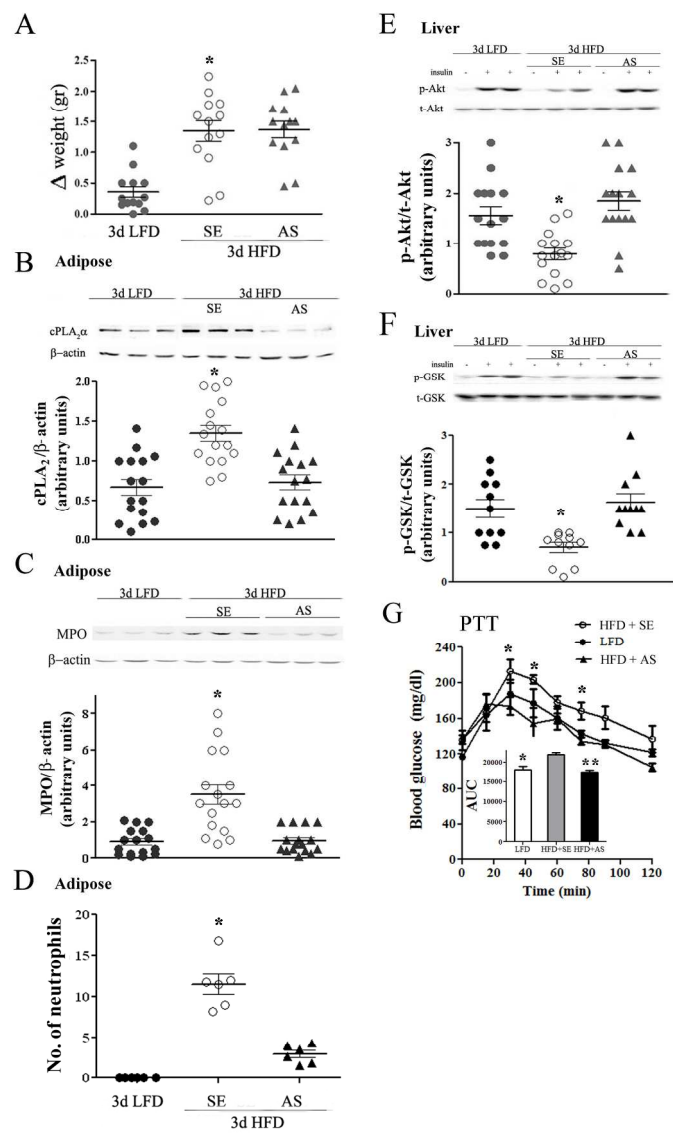
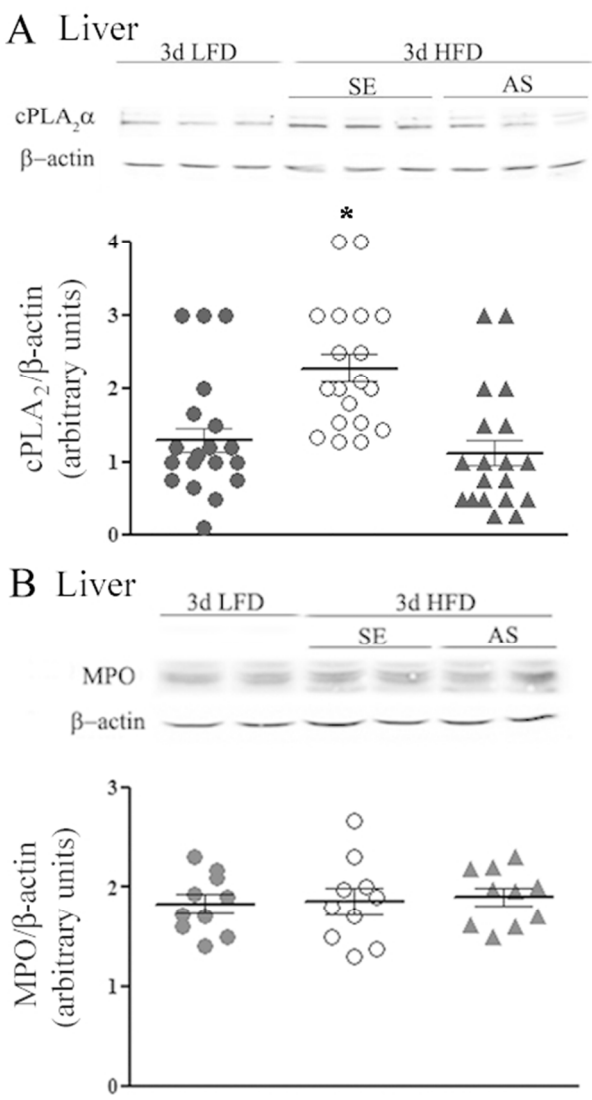


Figure 1

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140x229mm (300 x 300 DPI)



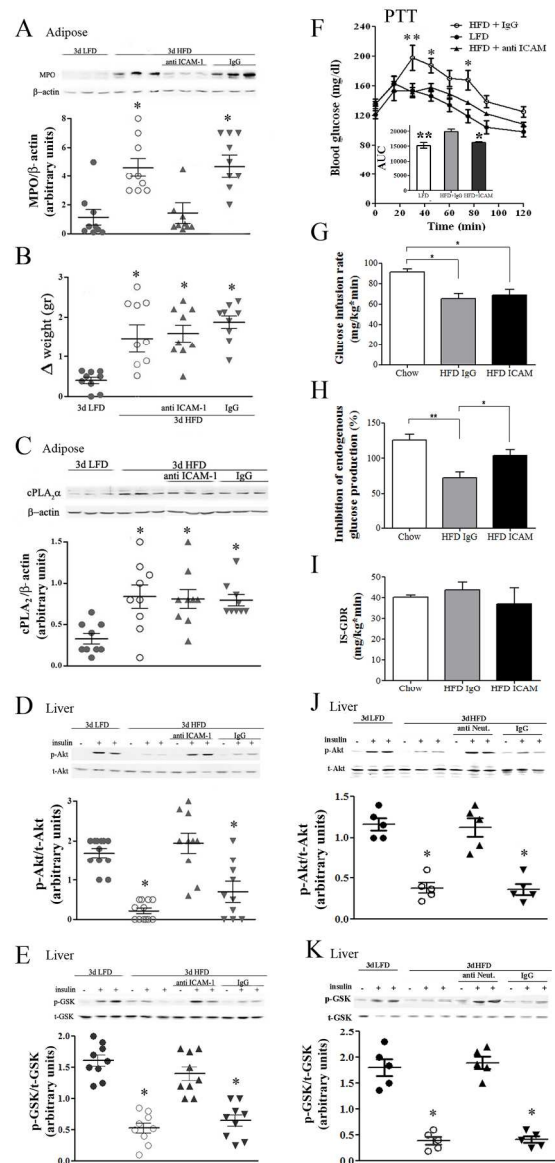


Figure 4

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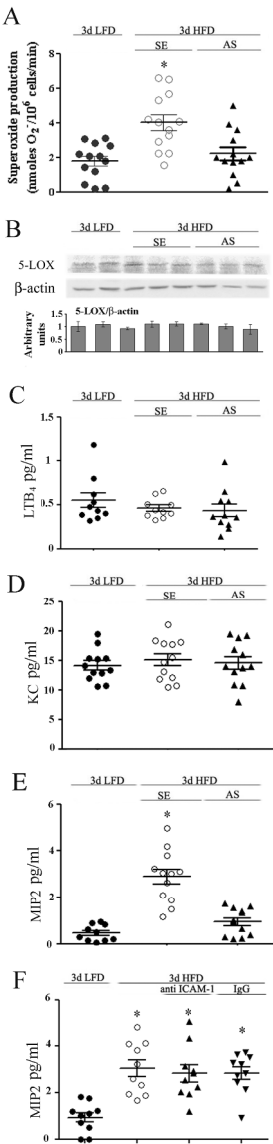


Figure 5

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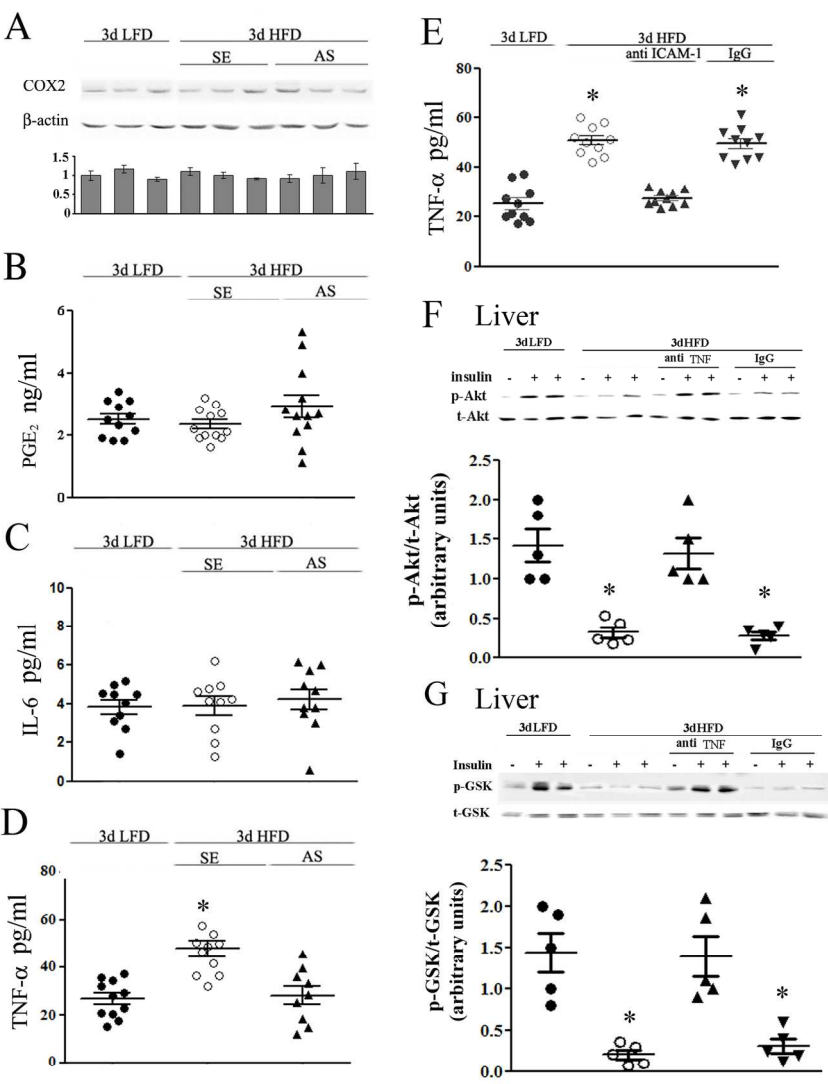
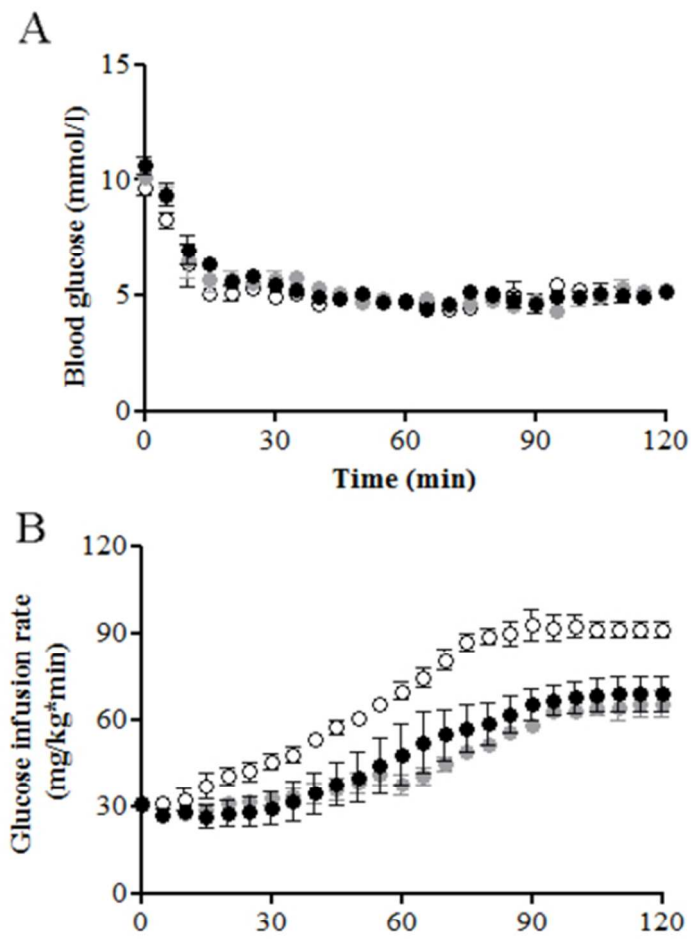


Figure 6

172x220mm (300 x 300 DPI)

Legends Supplement Figure 1

Blood glucose concentrations, glucose infusion rates and plasma insulin levels during hyperinsulinemic-euglycemic clamp (A) Blood glucose levels were clamped upon insulin infusion between 5 and 6 mmol/l. (B) In order to maintain euglycemia, glucose infusion rate was adjusted over time. ○ Chow-fed mice, ● IgG-treated HFD-fed mice, ● ICAM-treated HFD-fed mice. n = 4.



Supplemental Figure 1

43x66mm (300 x 300 DPI)